Transplant Immunology 23 (2010) 34–39

Contents lists available at ScienceDirect

Transplant Immunology

journal homepage: www.elsevier.com/locate/trim

Isatis tinctoria L. combined with co-stimulatory molecules blockade prolongs survival of cardiac allografts in alloantigen-primed mice

Xiangpeng Kang [a,1], Jibing Chen [b,1], Qing Qin [c,1], Feng Wang [d], Yongzhi Wang [d], Tianshu Lan [d], Shuo Xu [d], Feiyu Wang [d], Junjie Xia [d], Henrik Ekberg [e], Zhongquan Qi [d,*], Zhongchen Liu [a,*]

[a] The Surgery of Stomach Intestine, The Affilated Zhongshan Hospital of Xiamen University, Xiamen City, Fujian Province 361003, PR China
[b] Basic Medical Department of Medical College, Xiamen University, Fujian Province, PR China
[c] Dept. of Natural Products Chemistry, School of Pharmaceutical Sciences, Guangxi Medical University, Nanning, Guangxi Province, PR China
[d] Organ Transplantation Institute, Xiamen University, Xiamen City, Fujian Province 361005, PR China
[e] Malmö Hospital of Lund University, Malmö, Sweden

ARTICLE INFO

Article history:
Received 1 February 2010
Received in revised form 6 March 2010
Accepted 14 March 2010

Keywords:
Isatis tinctoria L.
Memory T cells
Alloantigen-primed mice

ABSTRACT

Memory T cells present a unique challenge in transplantation. Although memory T cells express robust immune responses to invading pathogens, they may be resistant to the effects of immunosuppressive therapies used to prolong graft survival. In previous studies, we found that compound K, the synthesized analogue of highly unsaturated fatty acids from *Isatis tinctoria* L., reduced acute cardiac allograft rejection in mice (Wang et al., 2009 [1]). Here, we further investigated the effect of compound K on cardiac allograft rejection in alloantigen-primed mice. We found that compound K significantly inhibited $CD4^+$ and $CD8^+$ memory T cells proliferation in a mixed lymphocyte reaction (MLR). *In vivo*, compound K combined with anti-CD154 and anti-LFA-1 monoclonal antibodies (mAbs) significantly extended the survival time of heart grafts in alloantigen-primed mice with no obvious toxic side effects. Furthermore, our data suggests that compound K works by reducing the expression of both IL-2 and IFN-γ within the graft rather than enhancing expression of regulatory T cells (Tregs). Compound K can also inhibit the alloresponses of memory T cells, while increasing the proportion of $CD4^+$ memory T cells in the spleen of the recipients and significantly reducing the level of alloantibodies in the serum. Our study highlights the unique immune effects of compound K that may be further explored for clinical use in extending the survival of transplant grafts.

1. Introduction

Memory T cells are important components of the immune system that defend against invading pathogens. In adults, 40–50% of the T cells circulating in the peripheral blood have memory phenotypes [2,3]. Exposure to alloantigens during previous transplantation, blood transfusions, or pregnancy, as well as continuous exposure to bacterial and viral pathogens, and cross-reactivity with allogenic MHC molecules may result in the development of alloreactive memory T cells in transplant patients [4–6]. It has been reported that high frequencies of alloreactive memory T cells in the peripheral blood of transplant patients are associated with poor allograft outcomes [7]. Memory T cells are not sufficiently inhibited by the clinical first-line immunosuppressive agents and monoclonal antibodies [8], which makes them the most difficult barrier to overcome in order to extend the graft survival time of the initial and secondary transplants. Other evidence have shown that memory B cells and alloantibodies also play an important role in transplantation [9].

We previously extracted the highly unsaturated fatty acids from *Isatis tinctoria* L., an herb used in traditional Chinese medicine, and used them as templates to synthesize various similar compounds. In previous studies, we studied one of those agents, compound K (K), and found that when combined with tacrolimus it could significantly reduce acute cardiac allograft rejection in mice. The mechanism of action of this combined treatment was postulated to be through inhibition of the secretion of IL-2 and IFN-γ by lymphocytes, rather than the activation of Tregs [1].

In this study, we investigated the effect and potential mechanism of compound K on cardiac allograft rejection in alloantigen-primed mice. We used combinations of compound K with antibody-mediated blockade of co-stimulatory molecules (CD154 and LFA-1) to demonstrate potential synergistic effects on long-term survival of heart grafts in alloantigen-primed mice.

2. Objectives

The objectives of this study were three-fold. We wanted firstly to determine whether the compound K could reduce memory T cell

* Corresponding authors. Qi is to be contacted at Tel.: +86 592 2180126. Liu, Tel.: +86 592 2992120.
E-mail addresses: oti@xmu.edu.cn (Z. Qi), zyj.tt@163.com (Z. Liu).
[1] These authors contributed equally to the study and share the first authorship.

0966-3274/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.trim.2010.03.006

proliferation in MLR; second, to determine whether compound K combined with anti-CD154 and anti-LFA-1 mAbs could reduce the accelerated rejection response in alloantigen-primed mice model; and third, to investigate the mechanism of action of compound K *in vivo*.

3. Materials and methods

3.1. Animals

Female C57BL/6 (B6) and BALB/c mice (8–12 weeks old) were purchased from Slac Laboratory Animal Co. Ltd. (Shanghai, China) and used as graft recipients and donors, respectively. Care and handling of animals were in accordance with the guidelines provided in the 'Guide for the Care and Use of Laboratory Animals' published by the U. S. Department of Health and Human Services.

3.2. Drugs and antibodies

The compound K, the analogue of highly unsaturated fatty acids from *Isatis tinctoria* L. was synthesized by associate professor Qin Qing from Guangxi Medical University (Guangxi, China). The following antibodies administered to the animals were produced from Bioexpress (West Lebanon): anti-CD154 (MR-1), anti-LFA-1 (M17/4), and their respective isotype controls. Antibodies used for FACS analysis were FITC anti-CD4 (GK1.5), FITC anti-CD8 (53-6.7), PECy5 anti-CD62L (MEL-14) and PE anti-CD44 (IM7), and their isotype controls (Biolegend, USA). Murine regulatory T cells were labeled using the Mouse Regulatory T cell staining kit from eBioscience (USA).

3.3. Murine cardiac transplantation model

Full-thickness BALB/c trunk skin tissues (circular pieces, diameter = 1.2 cm) were engrafted onto the lumbar region of B6 mice, and recipients that had rejected BALB/c skin 4 weeks after transplantation were defined as alloantigen-primed mice. Hearts from the donor mice were transplanted to the neck vessels of the alloantigen-primed B6 recipients using a non-suture cuff technique as described previously [10]. Graft survival was monitored by palpation (twice daily) and body weight was recorded daily for consecutive 15 days. B6 mice were treated with anti-CD154 (0.25 mg) + anti-LFA-1 (0.1 mg), compound K (10 mg/kg/day) or anti-CD154 (0.25 mg) + anti-LFA-1 (0.1 mg) + compound K (10 mg/kg/day). The antibodies were administered on the day of transplantation and 3 more times every alternate day. Compound K was given on days 0–10 post-transplantation, and the control group was given normal saline only.

3.4. Isolation of memory T cells

About 4 weeks after skin transplantation, the $CD4^+$ or $CD8^+$ memory T cells were autoMACS and FACS-purified from the spleens of the recipient mice. $CD4^+$ memory T cells were isolated by using the MagCellect Mouse Memory $CD4^+$ T Cell Isolation Kit (cat. no. MAGM206, R&D, USA). Briefly, splenocytes were incubated with a Biotin-antibody cocktail which targeted the unwanted cells, and then with microbead-conjugated anti-biotin mAb. The cell suspension was placed in magnetic field of a MACS separator. The supernatant in the tube is the final cell fraction containing the enriched $CD4^+$ Tm cells. Total $CD8^+$ T cells were also first isolated from spleen cells by using the MagCellect Mouse $CD8^+$ T Cell Isolation Kit (cat. no. MAGM 203, R&D, USA). These isolated $CD8^+$ T cells were then incubated with anti-CD8-FITC, anti-CD62L-PECy5 and anti-CD44-PE Ab and were sorted by FACS after gating on the $CD8^+CD44^{high}CD62L^{-/low}$ population. The purities of these cells were typically greater than 90% for $CD4^+$ memory T cells and greater than 95% for $CD8^+$ memory T cells detected by flow cytometry; the viability of these cells was greater than 90% detected by trypan blue staining.

3.5. Mixed lymphocyte reaction (MLR)

Memory T cells isolated from the spleen of the recipient mice using nylon wool columns (Wako, Japan) were used as responder cells. Spleen cells obtained from the BALB/c mice were used as stimulator cells. The responder cells (5×10^5 cells) were cultured in 96-well plates in the presence of stimulator cells (5×10^4 cells, pre-treated with mitomycin C, 40 μg/ml, Amresco, USA) in 200 μl RPMI 1640 supplemented with 10% FBS, penicillin, and streptomycin, and incubated at 37 °C in a 5% CO_2 humidified atmosphere for 72 h. Cell proliferation was measured using a bromodeoxyuridine (BrdU) cell proliferation assay kit (cat. no. 2750, Chemicon, USA). The measurements were performed in triplicates.

3.6. Pathological studies

Heart grafts ($n = 3$) were removed on day 5 post-transplantation. Part of each graft was used for quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR), and the rest was used for histological evaluation. Paraffin-embedded transventricular tissue sections (5 μm thick) were stained with hematoxylin and eosin (H&E). Graft rejection was graded on the basis of the extent of infiltration and the anatomical localization of inflammatory cells, according to the International Society of Heart and Lung Transplantation (ISHLT) standard [11,12]. Briefly, heart tissue was scored as follows: 0 = no damage; 1 (mild) = evidence of interstitial edema and focal necrosis; 2 (moderate) = graft displayed diffuse myocardial cell swelling and necrosis; 3 (severe) = necrosis with presence of contraction bands and neutrophil infiltrate, and 4 (highly severe) = widespread necrosis with presence of contraction bands, neutrophil infiltrate and hemorrhage.

3.7. Quantitative real-time reverse transcription-polymerase chain reaction

Grafts were removed from the recipients on day 5 after transplantation, and the RNA was extracted using Trizol (Invitrogen, USA). Reverse transcription and PCR were performed using ReverTra Ace® qPCR RT Kit (code no. FSQ-101) and SYBR® Green Realtime PCR Master Mix -Plus- (code no. QPK-212, 212T) (Toyobo, Japan), respectively. Analysis of the data was performed using the StepOne Real-Time PCR System (ABI, UK). β-actin was used as a normalizing control, and each reaction was performed in triplicates. The primer sequences for qRT-PCR are: β-actin forward 5′-CATCCGTAAAGACCTCTATGCCAAC-3′, and reverse 5′-ATGGAGCCACCGATCCACA-3′; IFN-γ forward 5′-CGGCACAGTCATTGAAAGCCTA-3′, and reverse 5′-GTTGCTGATGGCCTGATTGTC-3′; IL-2 forward 5′-GGAGCAGCTGTTGATGGACCTAC-3′, and reverse 5′-AATCCAGAACATGCCGCAGAG-3′; IL-10 forward 5′-GACCAGCTGGACAACATACTGCTAA-3′, and reverse 5′-GATAAGGCTTGGCAACCCAAGTAA-3′; Foxp3 forward 5′-CAGCTCTGCTGGCGAAAGTG-3′, and reverse 5′-TCGTCTGAAGGCAGAGTCAGGA-3′; TGF-β forward: 5′-TGACGTCACTGGAGTTGTACGG-3′, and reverse 5′-GGTTCATGTCATGGATGGTGC-3′.

3.8. Microlymphocytotoxicity test (MLC)

The MLC test was performed by adding the following reagents to each well of a 96-well plate: 5 μl mineral oil, 1 μl serum from the recipient mice, 1 μl guinea pig serum, and 1 μl of a BALB/c spleen cell suspension (2×10^3 cells/μl). After incubation at 22–25 °C for 45 min, the cells were stained using the Cell Viability Assay Kit (Beyotime Institute of Biotechnology, China) and the apoptosis level was calculated by counting random visual fields. In negative control wells, 1 μl of serum from naive mice was used instead of that from experimental groups. Each reaction was performed in triplicates.

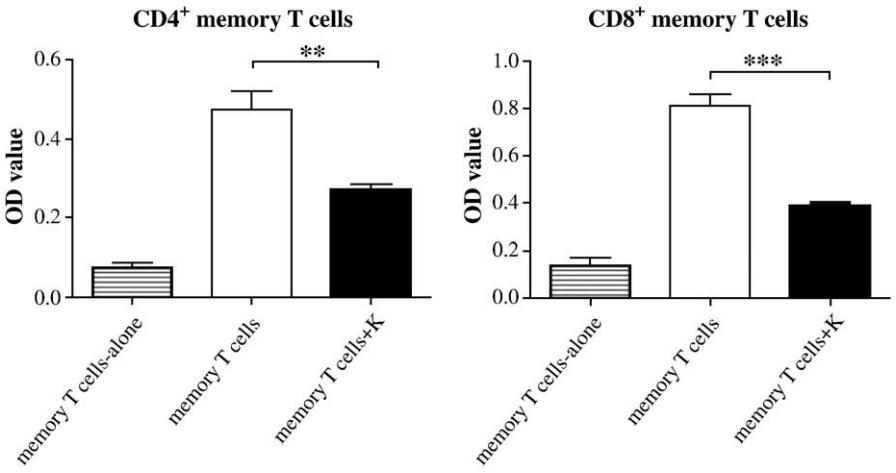

Fig. 1. Compound K reduces proliferation of alloreactive memory T cells in MLR assays. Responder and stimulator cells were mixed for 72 h. Cell proliferation was quantified using the BrdU method. Memory T cells alone were served as a negative control. Each group was tested in quadruplicate wells. Data are shown as mean ± SD and are representative of three separate experiments. Compared with the control group, both $CD4^+$ and $CD8^+$ memory T cells proliferation were inhibited by compound K (**$P<0.01$; ***$P<0.001$).

3.9. Statistical analysis

The median survival times (MSTs) of the four groups were calculated and compared by the Kaplan–Meier method. Data from the MLR, FACS and qRT-PCR experiments were analyzed by one-way analysis of variance (ANOVA) and expressed as mean ± standard deviation (SD). Because multiple comparisons were made during the analysis, a Bonferroni correction was calculated and applied. A P value <0.05 was considered statistically significant; $P<0.01$ and $P<0.001$ indicated highly significant differences. All analyses were performed using the GraphPad Prism® (GraphPad, Inc., CA) software.

4. Results

4.1. Effect of compound K on mixed lymphocyte response

We performed MLR experiments using compound K (80 μg/ml) to investigate whether it could inhibit the alloresponse levels of memory T cells. Proliferation of memory T cells was assessed after 72 h of culture using the BrdU method. We found that, compared with the control group, proliferation of both $CD4^+$ and $CD8^+$ memory T cells were both inhibited by compound K (Fig. 1).

4.2. Cardiac allograft survival time and body weight changes in graft recipients

In order to investigate whether the compound K combined with anti-CD154 and anti-LFA-1 mAbs can reduce the accelerated rejection response in an alloantigen-primed mice model, we divided the recipients into the following treatment groups: control group (normal saline); Ab group (anti-CD154 + anti-LFA-1); K group (compound K) and Ab + K group (anti-CD154 + anti-LFA-1 + compound K). As shown in Fig. 2, the MST was 3 days in the control group, 7 days in the Ab group, 4 days in the K group, and 12 days in the Ab + K group. The changes in body weight after transplantation were slight and mainly due to the surgical trauma (data not shown). These results suggest that the compound K combined with anti-CD154 and anti-LFA-1 mAbs can significantly prolong the graft survival time ($P<0.01$). Importantly, no side effects of compound K treatment were noted.

4.3. Compound K inhibits the accelerated rejection response in alloantigen-primed mice

At 5 days after the heart transplantation, the grafts were harvested and prepared for histology as outlined above. The ISHLT scores of the hearts harvested from the different treatment groups are shown in Fig. 3A. The grafts from the control group showed high levels of lymphocyte infiltration and severe tissue damage, while the grafts from the K and the Ab groups showed moderate lymphocyte infiltration and tissue damage. The grafts from the Ab + K group, however, showed only mild lymphocyte infiltration and tissue damage. These results suggest that treatment with compound K alone provides limited protection against the accelerated rejection responses, but that this protection is enhanced when combined with co-stimulatory molecules blockade.

4.4. Compound K reduces the expressions of both IL-2 and IFN-γ mRNA within the graft

The relative expressions of key cytokines mRNA in cardiac allografts were analyzed by qRT-PCR (Fig. 3B). Compared with the control group, the relative expressions of IL-2 and IFN-γ were significantly reduced in the K groups ($P<0.05$), and both transcripts were significantly less in the combined treatment group than in the Ab group ($P<0.001$). As seen in Fig. 3B, the expressions of IL-10 and Foxp3 were actually reduced in the compound K-treated group, compared with the control group ($P<0.01$). However, there was no difference in the relative expression of TGF-β among the four groups ($P>0.05$). These data suggest that compound K works by reducing the expression of both IL-2 and IFN-γ within the graft rather than enhancing expression of Tregs, resulting in prolonged graft survival times.

4.5. Compound K increased the proportion of $CD4^+$ memory T cells in recipients' spleens

Five days after heart transplantation, splenocytes were harvested and prepared for flow cytometry. We found that the number of $CD4^+CD44^{high}CD62L^{-/low}$ or $CD8^+CD44^{high}CD62L^{-/low}$ T cells in the Ab + K group was significantly lower compared with the Ab group ($P<0.05$); meanwhile, the percentage of $CD4^+CD44^{high}CD62L^{-/low}$ was higher in the K group than the control group (Fig. 4A; $P<0.05$). There was no difference in the percentage of $CD4^+Foxp3^+$ Tregs between the treatment group and the control group (Fig. 4B; $P>0.05$). These observations showed that the compound K combined with anti-CD154 and anti-LFA-1 mAbs can significantly inhibit the alloresponses of $CD4^+CD44^{high}CD62L^{-/low}$ and $CD8^+CD44^{high}CD62L^{-/low}$ T cells in spleens of alloantigen-primed mice. Furthermore, compound K increased the proportion of $CD4^+$ memory T cells in the spleens of the recipients.

4.6. Compound K inhibits the intensity of alloresponse of memory T cells in vitro

To assay the alloreactivity of T cells of spleen in recipients, the spleens were harvested 5 days post-heart transplantation, and the T cells were prepared for MLR. As is shown in Fig. 4C, compared with the control group, alloresponses of splenocytes were

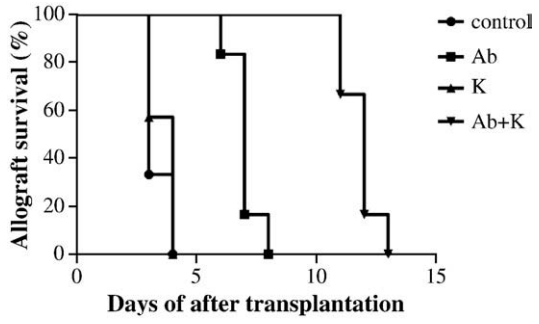

Fig. 2. Cardiac allograft survival time changes in graft recipients. Vascularized heterotopic heart transplants from BALB/c mice to B6 mice were monitored twice a day by manual palpation. Recipient mice were treated: control group (normal saline); Ab group (anti-CD154 + anti-LFA-1); K group (compound K) and Ab + K group (anti-CD154 + anti-LFA-1 + compound K). Kaplan–Meier curve of palpation scores for the four groups.

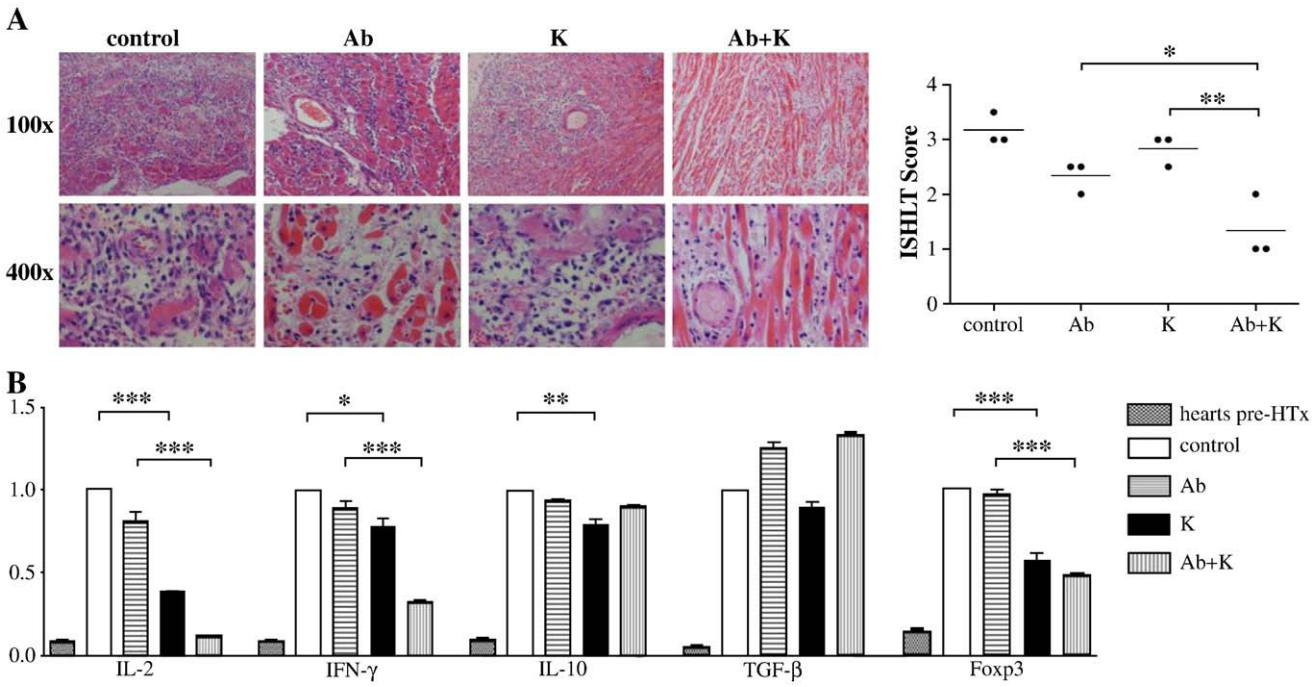

Fig. 3. Compound K inhibits the accelerated rejection response in alloantigen-primed mice and the relative expression of key cytokines mRNA within the allografts. Heart grafts were harvested on day 5 post-transplantation. Part of each graft was used for histological evaluation, and the rest was used for qRT-PCR analysis. (A) H&E stained heart tissues are shown on the left, the ISHLT scoring of the hearts are shown on the right. The dot plot represents the scores for each animal in each group. The line represents the mean scores ($n=3$, $^{*}P<0.05$; $^{**}P<0.01$). (B) The relative expression of key cytokines mRNA within the allografts. Each group was tested in quadruplicate wells. The levels of mRNA from hearts prior to transplantation were served as a negative control. Compared with the control group, the relative expressions of IL-2 and IFN-γ were significantly reduced in the K groups ($^{***}P<0.001$; $^{*}P<0.05$). Both IL-2 and IFN-γ expressions were significantly less in the combined treatment group than in the Ab group ($^{***}P<0.001$). The expressions of IL-10 and Foxp3 were actually reduced in the compound K-treated group, compared with the control group ($^{**}P<0.01$; $^{***}P<0.001$). There was no difference in the relative expression of TGF-β among the four groups ($P>0.05$). Data shown is the mean ± SD and is representative of three separate experiments.

significantly inhibited by compound K alone. Combination treatment with compound K and Ab also resulted in a significantly reduced alloresponses of splenocytes compared with the Ab group after 72 h of culture. It is notable that, although the compound K was able to inhibit the alloresponses of the splenocytes when compared with the control, it had an obvious synergistic effect when used in combination with anti-CD154 and anti-LFA-1 mAbs.

4.7. Combination treatment with compound K and Ab can reduce the level of alloantibodies in the serum of alloantigen-primed mice

The serum of alloantigen-primed mice was collected on day 5 after transplantation for MLC tests. Compared with the control group, the level of cell death rates was significantly lower in the combined treatment group (Fig. 5; $P<0.001$). Therefore, the combination treatment with compound K and Ab could reduce the level of alloantibodies in the serum of alloantigen-primed mice.

5. Discussion

Memory T cells are believed to be an important part of the barrier to extending the graft survival time of the transplantation in alloantigen-primed recipients [13–16]. It was reported that mycophenolate mofetil (MMF) [17] and some monoclonal antibodies such as anti-OX40L [18,19] and anti-CD122 [20] could inhibit memory T cells. *In vitro*, we found that compound K significantly inhibited proliferation of $CD4^+$ and $CD8^+$ memory T cells in MLR assays. Since we had also found in previous studies that compound K combined with tacrolimus significantly reduced acute cardiac allograft rejection in mice with no obvious toxic side effects, it seems that this compound displays strong immunosuppressive effects on effector and memory phenotype T cells and has the potential to be developed as a new immunosuppressant.

Here, we investigated the effect of compound K, either as a single treatment, or combined with co-stimulatory molecules blockade, on the survival time of heart grafts in alloantigen-primed mice. No side effects of compound K treatment on body weight were noted. Although the ease of inhibiting immune responses by blockade of T cell co-stimulation in naive rodent models, it is difficult to suppress those responses in animals with memory cells [21,22]. As shown in Fig. 2, the survival time of the cardiac allograft in the Ab group was limited (MST = 7 days); treatment with compound K alone extended cardiac allograft survival for only a short time over non-treated controls (4 days versus 3 days). Combined therapy (Ab + K) showed immunosuppressive effects comparable to non-treated controls (12 days versus 3 days). Notably, withdrawal of compound K 10 days after heart transplantation resulted in rapid graft rejection, possibly due to the short half-life of compound K (3 days), similar to the results found with FTY720 [23]. Mechanistically, however, compound K likely works in a different manner than FTY720. Zhang et al. indicated that FTY720 induces sequestration of circulating donor-reactive memory $CD4^+$ T cells in secondary lymphoid organs and synergizes with donor-specific transfusion (DST) and anti-CD154, leading to prolonged heart allograft survival in mice containing $CD4^+$ memory T cells [23]. Recently published data also suggested that the combination of FTY720 and DST/anti-CD154 promotes induction of $CD4^+CD25^+$ Tregs and results in long-term cardiac allograft survival in mice with interrupted CD62L-mediated lymph node homing [24]. In our study, we investigated the inhibitory mechanisms of compound K by examining histological sections of the allografts and by analyzing the levels of cytokines mRNA expression in the graft. We found that compound K inhibited IL-2 and IFN-γ gene expression when compared to the control group, and the combination of compound K and Ab had a synergistic inhibitory effect on the expression of IL-2 and IFN-γ that is more powerful than Ab alone. Compound K appeared not to induce IL-10, TGF-β and Foxp3 gene expression, but does inhibit IL-2 and IFN-γ gene expression. Our results suggest that the mechanism of compound K-induced tolerance in the accelerated rejection response in alloantigen-primed mice model involves reduction of the expression of both IL-

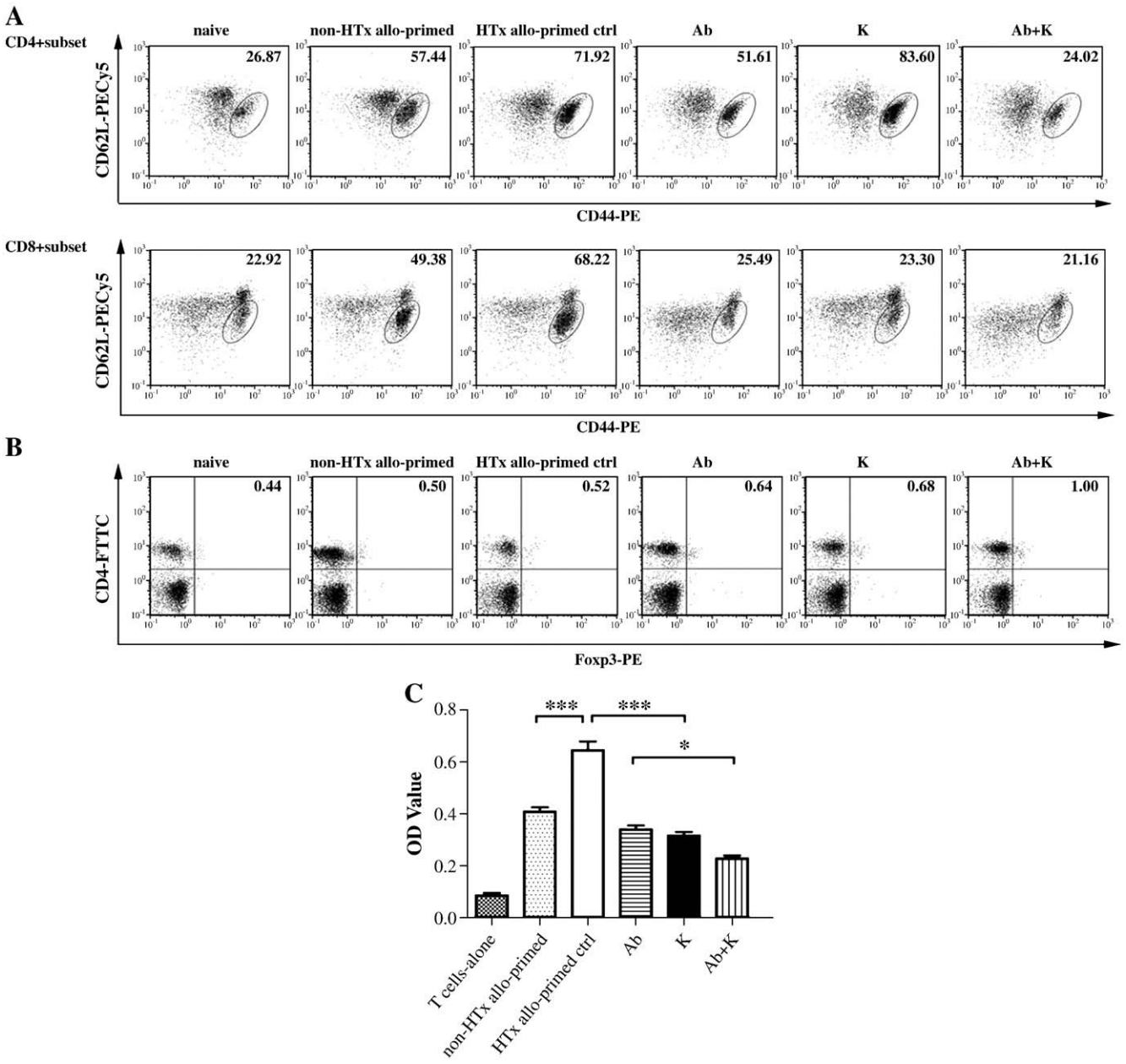

Fig. 4. Compound K effected the proportion of memory T cells (A) and Tregs (B) in the lymphocytes of the recipients' spleen and inhibited the intensity of alloresponses of the recipients' spleen T cells (C). The spleens were harvested 5 days post-heart transplantation, and the spleen lymphocytes and T cells were analyzed by flow cytometry and MLR, respectively. Data are representative of three separate experiments. (A) The percentage of $CD4^+CD44^{high}CD62L^{-/low}$ or $CD8^+CD44^{high}CD62L^{-/low}$ T cells in the Ab + K group was significantly lower compared with the Ab group ($P<0.05$); while the percentage of $CD4^+CD44^{high}CD62L^{-/low}$ was higher in the K group than the control group ($P<0.05$). (B) There was no difference in the percentage of $CD4^+Foxp3^+$ Tregs between the treatment group and the control group ($P>0.05$). (C) T cells lymphocytes isolated from the spleen of the recipient mice were used as responder cells. Spleen cells obtained from the BALB/c mice were used as stimulator cells. The responder cells (5×10^5 cells) were cultured in 96-well plates in the presence of stimulator cells (5×10^4 cells, pre-treated with mitomycin C) and cultured for 72 h. Cell proliferation was quantified using the BrdU method. Each group was tested in quadruplicate wells. The positive control group was given normal saline after transplantation; the responder cells alone were served as a negative control; the proliferative response of T cells from non-transplanted allo-primed mice was also shown. Compared with the positive control group, alloresponses were significantly inhibited by compound K alone (***$P<0.001$); combination treatment with compound K and Ab also resulted in a significantly inhibition of the alloresponses of splenocytes compared with the Ab group (*$P<0.05$).

2 and IFN-γ within the graft rather than inducing the proliferation of $CD4^+Foxp3^+$ Tregs. This notion is consistent with our previous finding in acute cardiac allograft rejection in mice [1].

Memory T cells mainly exist in the spleen and lymph node in alloantigen-primed mice. Homing of effector memory T cells especially to the spleen plays an important role in rejection of secondary transplantation [15,25]; therefore, we investigated the memory phenotype of the splenocytes 5 days after heart transplantation. As shown in Fig. 4A, the percentage of $CD8^+CD44^{high}CD62L^{-/low}$ T cells in the Ab group was lower compare to the control group, while there were no significant changes in $CD4^+CD44^{high}CD62L^{-/low}$ in mice treated with co-stimulatory molecules blocking Abs in comparison with the control group, consistent with results reported by Xu et al. in 2008 [26]. Furthermore, we found that the number of $CD4^+CD44^{high}CD62L^{-/low}$ or $CD8^+CD44^{high}CD62L^{-/low}$ T cells in the Ab + K group was significantly lower compared with the other groups, demonstrating that compound K combined with co-stimulatory molecules blockade can possibly inhibit the activation and/or proliferation of these memory T cells or induce apoptosis in these cells. Interestingly, the percentage of $CD4^+CD44^{high}CD62L^{-/low}$ T cells was higher in the K group than in the control

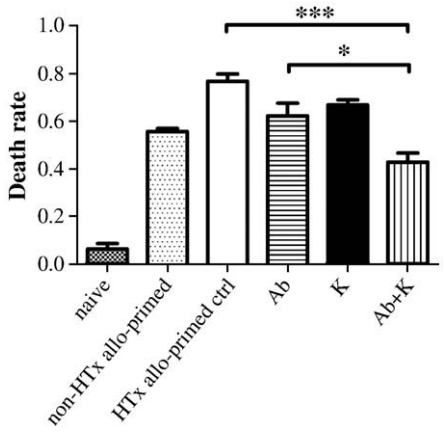

Fig. 5. Levels of alloantibodies in the serum of recipients. The serum of alloantigen-primed mice were collected at day 5 after transplantation for MLC assay. The positive control group was given normal saline after transplantation; and the serum from naive mice was served as a negative control. The serum from allo-primed non-transplanted mice was also shown. Each group was tested in quadruplicate wells. ($^{*}P<0.05$; $^{***}P<0.001$). Data are representative examples of three separate experiments.

group. Since compound K inhibits alloresponse *in vitro* [1] and co-stimulatory molecules blockade doesn't significantly suppresses homeostatic proliferation [27], it would seem to indicate there is homeostatic proliferation following depletion of effector T cells by compound K, similar to the report by Pearl et al. [28]. Furthermore, the MLR assays for alloreactivity of T cells of spleen in recipients showed that, compared with the control group, alloresponses of splenocytes were significantly inhibited by compound K alone, while the combination treatment with compound K and Ab also resulted in a significant inhibition of the alloresponses of splenocytes after 72 h of culture. It is notable that compound K inhibited both effector and memory T cells while, resulting in homeostatic proliferation of memory T cells in the spleens of recipients.

It is important to define the memory B cells, as well as pre-existing alloantibodies in prolonging the survival time of secondary allografts in alloantigen-primed models [29,30]. But the impact of memory B cells and alloantibodies on the ability to induce transplantation tolerance has not been elucidated [31]. As we found that combination treatment of compound K and Ab could reduce the level of alloantibodies in the serum of alloantigen-primed mice, our data may provide insight into the roles of memory B cells and alloantibodies in graft acceptance.

In summary, the synthesized analogue of highly unsaturated fatty acid compound K from *Isatis tinctoria* L. has low toxicity and, combined with anti-CD154 and anti-LFA-1 mAbs, can significantly prolong the survival time of heart grafts in alloantigen-primed mice. Our findings emphasize that compound K works by reducing the expression of both IL-2 and IFN-γ within the graft, rather than enhancing expression of Tregs, to allow prolonged graft survival times. Furthermore, compound K inhibited the *in vitro* alloresponses of memory T cells, while increasing the proportion of $CD4^{+}$ memory T cells in the spleen of recipients. Together, these data suggest that compound K uniquely inhibits memory T cells and reduces alloantibidies, but further studies are needed to expand on these findings.

Acknowledgement

This study was supported by a research funding from the Technological Project of Xiamen City (no. 3502Z 20084013).

References

[1] Wang Y, Qin Q, Chen J, et al. Synergistic effects of *Isatis tinctoria* L. and tacrolimus in the prevention of acute heart rejection in mice. Transpl Immunol 2009;22:5–11.
[2] Douek DC, McFarland RD, Keiser PH, et al. Changes in thymic function with age and during the treatment of HIV infection. Nature 1998;396:690–5.
[3] McFarland RD, Douek DC, Koup RA, Picker LJ. Identification of a human recent thymic emigrant phenotype. Proc Natl Acad Sci U S A 2000;97:4215–20.
[4] Burrows SR, Khanna R, Burrows JM, Moss DJ. An alloresponse in humans is dominated by cytotoxic T lymphocytes (CTL) cross-reactive with a single Epstein–Barr virus CTL epitope: implications for graft-versus-host disease. J Exp Med 1994;179:1155–61.
[5] Burrows SR, Silins SL, Khanna R, et al. Cross-reactive memory T cells for Epstein–Barr virus augment the alloresponse to common human leukocyte antigens: degenerate recognition of major histocompatibility complex-bound peptide by T cells and its role in alloreactivity. Eur J Immunol 1997;27:1726–36.
[6] Taylor DK, Neujahr D, Turka LA. Heterologous immunity and homeostatic proliferation as barriers to tolerance. Curr Opin Immunol 2004;16:558–64.
[7] Heeger PS, Greenspan NS, Kuhlenschmidt S, et al. Pretransplant frequency of donor-specific, IFN-gamma-producing lymphocytes is a manifestation of immunologic memory and correlates with the risk of posttransplant rejection episodes. J Immunol 1999;163:2267–75.
[8] Valujskikh A, Li XC. Frontiers in nephrology: T cell memory as a barrier to transplant tolerance. J Am Soc Nephrol 2007;18:2252–61.
[9] Sanz I, Wei C, Lee FE, Anolik J. Phenotypic and functional heterogeneity of human memory B cells. Semin Immunol 2008;20:67–82.
[10] Matsuura A, Abe T, Yasuura K. Simplified mouse cervical heart transplantation using a cuff technique. Transplantation 1991;51:896–8.
[11] Billingham ME, Cary NR, Hammond ME, et al. A working formulation for the standardization of nomenclature in the diagnosis of heart and lung rejection: Heart Rejection Study Group. The International Society for Heart Transplantation. J Heart Transplant 1990;9:587–93.
[12] Aneja R, Hake PW, Burroughs TJ, et al. Epigallocatechin, a green tea polyphenol, attenuates myocardial ischemia reperfusion injury in rats. Mol Med 2004;10:55–62.
[13] Brook MO, Wood KJ, Jones ND. The impact of memory T cells on rejection and the induction of tolerance. Transplantation 2006;82:1–9.
[14] Neujahr D, Turka LA. Lymphocyte depletion as a barrier to immunological tolerance. Contrib Nephrol 2005;146:65–72.
[15] Yang J, Brook MO, Carvalho-Gaspar M, et al. Allograft rejection mediated by memory T cells is resistant to regulation. Proc Natl Acad Sci U S A 2007;104:19,954–9.
[16] Moxham VF, Karegli J, Phillips RE, et al. Homeostatic proliferation of lymphocytes results in augmented memory-like function and accelerated allograft rejection. J Immunol 2008;180:3910–8.
[17] Trzonkowski P, Zilvetti M, Chapman S, et al. Homeostatic repopulation by CD28–CD8+ T cells in alemtuzumab-depleted kidney transplant recipients treated with reduced immunosuppression. Am J Transplant 2008;8:338–47.
[18] Prell RA, Evans DE, Thalhofer C, et al. OX40-mediated memory T cell generation is TNF receptor-associated factor 2 dependent. J Immunol 2003;171:5997–6005.
[19] Dawicki W, Bertram EM, Sharpe AH, Watts TH. 4-1BB and OX40 act independently to facilitate robust CD8 and CD4 recall responses. J Immunol 2004;173:5944–51.
[20] Minamimura K, Sato K, Yagita H, et al. Strategies to induce marked prolongation of secondary skin allograft survival in alloantigen-primed mice. Am J Transplant 2008;8:761–72.
[21] Valujskikh A, Pantenburg B, Heeger PS. Primed allospecific T cells prevent the effects of costimulatory blockade on prolonged cardiac allograft survival in mice. Am J Transplant 2002;2:501–9.
[22] Onodera K, Chandraker A, Schaub M, et al. CD28-B7 T cell costimulatory blockade by CTLA4Ig in sensitized rat recipients: induction of transplantation tolerance in association with depressed cell-mediated and humoral immune responses. J Immunol 1997;159:1711–7.
[23] Zhang Q, Chen Y, Fairchild RL, Heeger PS, Valujskikh A. Lymphoid sequestration of alloreactive memory CD4 T cells promotes cardiac allograft survival. J Immunol 2006;176:770–7.
[24] Ochando JC, Yopp AC, Yang Y, et al. Lymph node occupancy is required for the peripheral development of alloantigen-specific Foxp3+ regulatory T cells. J Immunol 2005;174:6993–7005.
[25] Surh CD, Sprent J. Homeostasis of naive and memory T cells. Immunity 2008;29:848–62.
[26] Xu H, Yan J, Huang Y, et al. Costimulatory blockade of CD154-CD40 in combination with T-cell lymphodepletion results in prevention of allogeneic sensitization. Blood 2008;111:3266–75.
[27] Wu Z, Bensinger SJ, Zhang J, et al. Homeostatic proliferation is a barrier to transplantation tolerance. Nat Med 2004;10:87–92.
[28] Pearl JP, Parris J, Hale DA, et al. Immunocompetent T-cells with a memory-like phenotype are the dominant cell type following antibody-mediated T-cell depletion. Am J Transplant 2005;5:465–74.
[29] Nagumo H, Abe J, Kano H, et al. Distinct response in maintenance of human naive and memory B cells via IL-21 receptor and TCL1/Akt pathways. Cell Immunol 2009;256:56–63.
[30] Coquet JM, Kyparissoudis K, Pellicci DG, et al. IL-21 is produced by NKT cells and modulates NKT cell activation and cytokine production. J Immunol 2007;178:2827–34.
[31] Burns AM, Ma L, Li Y, et al. Memory alloreactive B cells and alloantibodies prevent anti-CD154-mediated allograft acceptance. J Immunol 2009;182:1314–24.